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(54) Assay systems utilising more than one enzyme.

(57) This specification discloses assay systems which detect the presence of, or measure or monitor the extent of, an enzyme-catalysed reaction on a substrate. The systems of the present invention can be used to detect or measure the enzyme or the substrate, depending upon circumstances.

The specification further discloses a method of assay of the type in which an electrode (1) poised at a suitable potential is contacted with a system comprising a first enzyme, (GO) a substrate (G) which undergoes a reaction catalysed by the said enzyme, and a mediator compound (F) which transfers charge to the electrode (1) from the first enzyme (GO) when it is catalytically active, whereby the current flowing in the electrode is a measure of the reaction taking place; in which at least one further enzyme (HK) and associated compound (ATP) is incorporated into the system, the further enzyme (HK) being productive of, or also being reactive with, the substrate (G) so as to affect its presence or level, but not being electrochemically linked by the mediator (F) to the electrode (1), whereby the consequent difference in electrode current flowing with, and in the absence of, the second enzyme (HK) and its associated compound is a measure of the extent of reaction of the further enzyme with its associated compound and thus permits the amount of one to be established if the amount of the other is known.

There are two main subdivisions of the present disclosure (i) in which the electrode is provided at its surface with the first enzyme and the mediator compound to constitute a sensor electrode and (ii) in which the further enzyme exerts a catalytic change on a specific further substrate to yield substrate for the first enzyme.

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Title:- ASSAY SYSTEMS UTILISING MORE THAN ONE ENZYME

This invention relates to essay systems which detect the presence of, or measure or monitor the extent of, an enzyme-catalysed reaction on a substrate. The systems of the present invention can be used to detect or measure the enzyme or the substrate, depending upon circumstances.

Our European Patent Application 82305598 describes and claims a sensor electrode which comprises at least at an extreme surface thereof a combination of an enzyme and a mediator compound which transfers charge to the electrode when the enzyme is catalytically active. Such an electrode, when contacting the specific substrate for the enzyme and poised at a suitable potential gives a signal responsive to the presence of, or indicative of the extent of, the enzyme/substrate reaction, even in a complex mixture of substrates since the enzyme is specific to the desired substrate component.

The practical operation of such a system depends on the incorporation of the mediator compound. A number of types of such compounds are disclosed in that application, such as polyviologens, fluoranil,

(c) the pH-independent redox potential and the slow autoxidation of the reduced form.

These compounds lend themselves to the formation of derivatives, e.g. by substitution of one or both cylopentadienyl rings and/or by polymerisation. We have studied a number of derivatives of ferrocene such as those listed in the table below;

Ferrocene derivative	Eo	Solubility	E
1,1'-dimethyl-	100	I,D	-
acetic acid	124	S	370
hydroxyethyl-	161	S	-
ferrocene	165	I,D	335
1,1'bis(hydroxymethyl)-	224	S	385
monocarboxylic acid	275	S	420
1,1'-dicarboxylic acid	285	S	-
chloro-	345	I,D	-
methyl trimethylamino-	400	S	-

S indicates water solubility; I,D mean respectively insoluble and detergent solubilised in 3% Tween-20. E^{o} is in mV vs a standard calomel electrode, E is measured in $cm^{-1}M^{-1}$.

The E^{o} values of various ferrocenes in phosphate buffer at pH 7.0 given in the above table, span a range

Enzyme	Substrate
Flavo-proteins	
Pyruvate Oxidase	Pyruvate
L-Amino Acid Oxidase	L-Amino Acids
Aldehyde Oxidase	Aldehydes
Xanthine Oxidase	Xanthines
Glucose Oxidase	Glucose
Glycollate Oxidase	Glycollate
Sarcosine Oxidase	Sarcosine
Lactate Oxidase	Lacate
Glutathione reductase	NAD(P)H
Lipoamide Dehydrogenase	NADPH
PQQ Enzymes	
Glucose Dehydrogenase	Glucose
Methanol Dehydrogenase	Methanol and other Alkanols
Methylamine Dehydrogenase	Methylamine
Haem-Containing Enzymes	
Lactate Dehydrogenase (Yeast Cytochrome B2)	Lactate
Horse-radish Peroxidase	Hydrogen Peroxide
Yeast Cytochrome C Peroxidase	Hydrogen Peroxide
Metalloflavoproteins	
Carbon monoxide Oxidoreductase	Carbon Monoxide
Cuproproteins	
Galactose Oxidase	Galactose

have been shown to give useful readout signals when incorporated into such systems.

and in the absence of, the second enzyme and its associated compound is a measure of the extent of reaction of the further enzyme with its associated compound and thus permits the amount of one to be established if the amount of the other is known.

Our copending application of even date entitled "Analytical Equipment and Sensor Electrodes therefor" describe the nature and manufacture of sensor electrodes. Such electrodes are preferred in the practice of the present invention in which preferably the electrode is provided at its surface with the first enzyme and the mediator compound to constitute a sensor electrode.

However, our further copending application of even date entitled "Assay techniques utilising Specific Binding Systems" and relating to specific binding agents (antibodies, or nucleic acid sequences) and their effect on the enzymes or mediators as an assay tool, describes systems in which the electrode can be a clean carbon rod, or having only the mediator, or only the enzyme, or occasionally include a substrate. Such systems can be incorporated into the present invention if desired, which is not limited to sensor electrodes per se but is concerned with the overall method of assay irrespective of the details of the embodying system.

compounds hexokinase and ATP, used in a combination where the amount of one is known, which comprises :

(a) contacting with a solution of glucose an electrode having at its surface a glucose oxidoreductase and a mediator compound to transfer charge from the said enzyme to the electrode when the enzyme is catalytically active, thereby to set up a steady electrode current based on the glucose level,

(b) adding to the solution the hexokinase and ATP, so as to set up with the glucose a competitive phosphorylation to which the electrode is insensitive, whereby the steady current is correspondingly reduced and,

(c) deriving a value for te unknown level from the rate of, extent of, or glucose compensation for, the reduction in current.

Another specifically valuable form of the invention consists in a method of assay for creatine kinase, which comprises:

(a) contacting with a mixed solution of hexokinase, adenosine disphosphate, creatine phosphate and glucose an electrode having at its surface a glucose

transfer charge from the enzyme to the electrode when the enzyme is catalytically active, thereby to set up a steady electrode current depending on the available glucose level remaining after the competitive ATP/hexokinase phosphorylation reaction to glucose-6-phosphate to which the electrode is insensitive, whereby the steady current is correspondingly reduced;

(b) adding to the solution creatine to be assayed under conditions in which ATP is converted to ADP, with the formation of creatine phosphate, by the creatine kinase, and thereby decreases the amount of ATP available fo the competitive glucose phosphorylation reaction,

(c) deriving a value for the unknown creatine level from the rate of, or extent of alteration in electrode current.

The second subdivision of the invention lends itself to the fabrication of rather more elaborate electrodes. Thus, the electrode itself may be provided with the first enzyme, the mediator compound therefor, and the further enzyme to constitute a sensor electrode for the said further substrate.

For instance, the electrode may be coated with sarcosine

The invention will be further described with reference to the accompanying drawings, in which :-

Figure 1 shows a scheme of competitive glucose oxidase/glucose and hexokinase/glucose reactions, used to detect or measure ATP or hexokinase;

Figure 2 shows a similar reaction further modified to detect or measure creatine kinase;

Figure 3 shows a similar reaction further modified to detect or measure creatine;

Figure 4 shows a scheme of reaction to detect or assay creatinine;

Figure 5 shows graphically the effect of adding creatine kinase to a steady state solution as described with reference to Figure 2; and

Figures 6 to 13 show graphically voltammograms and derived graphical results from specific experiments carried out.

A glucose sensor electrode was made up starting from a carbon rod. On this was deposited a solution of 1,1'-dimethylferrocene in toluene. The toluene was

this glucose which can either react with the glucose oxidase GO to provide electron transfer i.e. charge transfer via the ferrocene to the electrode (as before) or react with added hexokinase enzyme (HK) and adenosine triphosphate (ATP), a reaction which is not linked with the electrode. In the first case glucose oxidation products are produced, such as glyceraldehyde or gluconolactone depending on the enzyme; in the second, glucose-6-phosphate, with the ATP being converted to ADP (adenosine diphosphate). The higher the concentrations of ATP and HK, the greater the extent to whch this competing reaction decreases the expected reading of electrode current. If the HK concentration is known, the ATP concentration can be deduced, and vice versa. Of course, the electrode could also be used for a qualitative indication, i.e. mere detection of the HK/ATP presence.

Figure 2 illustrates another possible configuration of the assay system using an electrode made up as before. In this embodiment the assay measures the concentration of the enzyme creatine kinase (CK) (E.C. 2.7.3.2) which converts the phosphoguanidine, creatine phosphate, CP to creatine CN (The system could equally well assay enzymes which have as substrates phosphoguanidines other than creatine phosphate, such as arginine phosphate; or by a suitable selection of enzyme the system could assay

to reach a steady state and comparing a final current reading with an initial current reading. Figure 5 illustrates how the initial steady catalytic current obtained with a system such as that described above with reference to Figure 2 falls when a solution containing creatine kinase is added to the system.

The creatine kinase assay give a linear response in the range 10-10^4 Units/litre and can therefore be used in the diagnosis of a wide range of conditions, for example myocardial injuries (such as acute myocardial infarction or facultative myocardial injury) delirium tremens and muscular dystrophy, all of which cause elevated creatine kinase levels in the blood.

Figure 3 illustrates a further possible embodiment which may be employed to detect the substrate creatine. In this embodiment which is buffered at pH 9, the direction of the reaction catalysed by creatine kinase is reversed.

In operation, a steady-state system is prepared as before, but with glucose (G), hexokinase (HK), creatine kinase (CK) and adenosine triphosphate (ATP). When a glucose 1,1'-dimethylferrocene electrode as described above is introduced into the solution, the steady-state electrode current will be less than that available with the glucose level above, because the adenosine

(cc $\geq$ 0.99) with the activity of the enzyme in plasma.

Various modifications may be made within the scope of this embodiment. For example, a further enzyme may be incorporated to convert creatinine to creatine thus allowing assay systems to be constructed for creatinine, although Fig. 4 below shows an alternative assay system for creatinine.

Figure 4 shows a different type of assay technique which does not depend on competitive reaction for substrate, and which utilises different enzymes suitable for the assay of glycine derivatives.

In the operation of the method shown in Fig. 4 an electrode is made up as described above, and in our corresponding applications incorporated herein by way of reference; but instead of immobilising glucose oxidase on the electrode surface there is immobilised on the surface a mixture of sarcosine oxidase, creatininase (creatinine aminohydrolase) and creatinase (creatine amidinohydrolase).

Sarcosine (N-methylglycine) is an intermediate in the metabolism of one-carbon compounds, and a constituent of the actinomycins, a potent group of antimetabolites.

Example 1

To the working compartment of a two-compartment electrochemical cell incorporating a 4mm diameter gold working electrode, a 0.5cm^2 platinum gauze counter electrode and a saturated calomel reference electrode (SCE), separated from the working compartment by a Luggin capillary, was added 1ml of Tris/HCL buffer (50mM, pH 7.5) containing ferrocene monocarboxylic acid (200 μM).

Figure 6 shows a D.C. cyclic voltammogram obtained at a scan rate of 5mVs^{-1} over the range 0 to +500 mV vs. SCE. The forward and reverse electrochemical waves are consistent with the reversible couple $E_{1/2}$ (Ferrocene monocarboxylic acid/ferricinium monocarboxylic acid) = +275 mV vs. SCE, where $E_{1/2}$ is the half wave potential of the system.

Subsequent serial addition of sarcosine dehydrogenase 50 IU ml^{-1} and creatinine amidohydrolase 50 IU ml^{-1} had no effect upon the reversible electrochemistry of ferrocene-monocarboxylic acid, curve (a).

However, upon further addition of creatinase (5mM) voltammogram (b) was obtained. The enhanced anodic current is indicative of a catalytically coupled

creatine kinase, [EC 2.7.3.2] (CK), constitutes ca. 20% of the soluble sarcoplasmic protein of heart muscle. Consequently, the occurance of elevated levels of CK in the blood often results in the diagnosis of acute myocardial infarction (AMI).

Several methods for measuring CK activity in plasma have been devised and are in daily use in clinical biochemistry laboratories, to perform over 30 million determinations per year.

Creatine kinase catalyses the reversible transfer of a phosphate residue from adenosine-5'-triphosphate (ATP) to creatine.

The reaction product, creatine phosphate, represents an essential energy store for contraction, relaxation and transport of substances within muscle cells.

Creatine kinase is a dimeric enzyme, molecular weight 82 000, constituted of two subunits weighing 41 000 (3). In human tissue two different types of subunits exist, designated M (muscle) and B (brain). The dimeric enzyme can have the following forms: CK-MM skeletal muscle type, CK-BB brain type and CK-MB myocardial type. These isoenzymes can be separated by electrophoretic techniques or by an immuno-inhibition

Condition	Total CK activity U/L	CK-MB activity U/L
Neurosurgical operations	63-610	0-88
Weight lifters	110-740	0-10
Polytrauma without myocardial injury	76-6220	0-230

The rapid response time of the ferrocene-based glucose enzyme electrodes indicates that in addition to monitoring bulk glucose concentrations, the device could be used to monitor the rates of change in bulk glucose concentrations. Thus CK activity could be determined using the coupled reactions sequence shown e.g. in Fig. 2 with the glucose enzyme electrode monitoring the rate of consumption of glucose. Under optimised conditions the rate of decrease in the electrode current should be proportional to the rate of consumption of glucose, which in turn would be proportional to the rateof consumption of creatine phosphate, from which the activity of CK can be estimated.

(b) Reagents

Creatine phosphate, adenosine-5'-diphosphate, adenosine-5'-triphosphate, creatine kinase from rabbit muscle with an activity of 800 IU mg^{-1} at 37°C and hexokinase from yeast with an activity of 1600 IU ml^{01}

Clinical Biochemistry Laboratory of the John Radcliffe Hospital, Oxford.

(e) Uncoupling the glucose oxidase reaction

Figure 7 shows at (a) a D.C. cylic voltammogram of ferrocene monocarboxylic acid in 25mM Tris-HCl buffer, pH 7.0, containing 20mM magnesium chloride and 10mM glucose. Addition of glucose oxidase, gave curve (b) showing a typical catalytic current at oxidising potentials, resulting from the enzymatically coupled oxidation of glucose. When hexokinase 20 IU ml^{-1}, is added, no change in the voltammogram is observed. However, upon addition of ATP to a final concentration of 10mM, the catalytic behaviour is no longer observed and the voltammogram associated with reversible electrochemistry of the ferrocene is again obtained, at at (a). These observations are consistent with phosphorylation of glucose to form glucose-6-phosphate, thus removing from solution the substrate for the electrochemically coupled oxidation reaction.

Since none of the components of the system interfered with the electrochemistry of ferrocene monocarboxylic acid, or showed any direct electrochemistry over the range of potential scanned, 0-450mV vs SCE, it was possible to investigate the response of the glucose

reagent concentrations were found to be in sufficient excess to ensure that the rate of glucose consumption in the assay was limited by the activity of creatine kinase. When creatine kinase, with a reported activity of 500 IU l^{-1} is added to the system, the current decreases with time. From the initial rate of decrease, the rate of glucose consumption was calculated. The rate of glucose consumption should be equivalent to the activity of creatine kinase in μmoles creatine phosphate consumed min^{-1} mg^{-1}. From the results a correlation coefficient of 0.99 was calculated. (Fig. 12).

(h) Detection limits of Creatine kinase assay

In post-AMI patients, plasma CK activities in the range 30-2000 IU l^{-1}, are common. The assay procedure, when investigated in buffered solutions, is accordingly expected to be suitable for monitoring clinical levels of CK (see Fig. 12). The upper limit of the electrode response is ca. 10 IU ml^{-1} (10 000 IU l^{-1}).

(i) Assay of creatine kinase in plasma samples

It was not possible to obtain authentic plasma samples from post-AMI patients, so samples were simulated by adding clinically relevant concentrations of CK to human plasma. Aliquots of ADP, $MgCl_2$ and HK were also added

TABLE II

CK Added IU/L	Buffer pH 7.0 IU/L	Plasma pH 7.7 IU/L	Plasma corrected to pH 7.0 IU/L
150	152	64.7	154
150	152	66.8	159
150	148	59.6	142
90	83	37.0	88
70	76	31.5	75
30	31	2.5	5.9
15	15	1.9	4.5
7.5	7.3	2.3	5.3
7.5	7.3	3.6	8.6

3. A method as claimed in claim 1 in which the further enzyme exerts a competitive reaction on the substrate and thus leads to a decrease in current flowing in the electrode.

4. A method as claimed in claim 3 in which (a) a sensor electrode provided at its surface with the first enzyme and with the mediator compound is contacted with a substrate to give a steady current reading (b) the second enzyme and associated compound one of which is in unknown quantity are added to set up a competitive reaction and hence decrease the electrode current and (c) the rate or extent of decrease, or the extent of substrate addition necessary to compensate for the decrease in electrode current, is noted as a measure of the mount of unknown component.

5. A method as claimed in claim 3 or 4 in which the further enzyme is a kinase and the associated compound is a high energy phosphate.

6. A method as claimed in claim 3 or 4, in which the further enzyme is a hexokinase and the associated compound is ATP.

7. A method of assay for the unknown one of the pair of compounds hexokinase and ATP, used in a combination

electrode current based on the glucose level,

(b) adding to the solution creatine kinase to be assayed, under conditions in which adenosine diphosphate (ADP) is converted to adenosine triphosphate (ATP) by reaction of the creatine phosphate and so that consequentially the glucose is reacted, in competition with the oxidoreductase enzyme reaction, to glucose-6-phosphate by the hexokinase and ATP phsophorylation to which the electrode is insensitive, whereby the steady current is correspondingly reduced, and

(c) deriving a value for the unknown creatine kinase level from the rate of, extent of, or glucose compensation for, the reduction in current.

9. A method of assay for creatine which comprises :
(a) contacting with a mixed solution of hexokinase, adenosine triphosphate (ATP), creatine kinase and glucose an electrode having at its surface a glucose oxidoreductase enzyme and a mediator compound to transfer charge from the enzyme to be electrode when the enzyme is catalytically active, thereby to set up a steady electrode current depending on the available glucose level remaining after the competitive ATP/hexokinase phosphorylation reaction to

contacted with a solution containing creatine for assay thereof by conversion to sarcosine.

13. A method as claimed in claim 11 in which the electrode is coated with sarcosine oxidase or dehydrogenase, creatinase, creatininase, and a mediator to transfer charge from the sarcosine to the electrode, and is contacted with creatinine for assay thereof by serial conversion to creatine and sarcosine.

14. A method as claimed in claim 1, 2, 3 ,4, 7, 8 or 9 in which the first enzyme is a glucose oxidase.

15. A method as claimed in claim 1, 2, 3, 4, 7, 8 or 9 in which the first enzyme is a glucose dehydrogenase.

16. A method as claimed in claim 1, 2, 3, 4, 7 8, 9, 10, 11, 12 or 13 in which the mediator is a ferrocene.

17. A method as claimed in claim 1, 2, 3, 4, 7, 8, 9, 199, 11, 12 or 13 in which the mediator is 1,1'-dimethylferrocene.

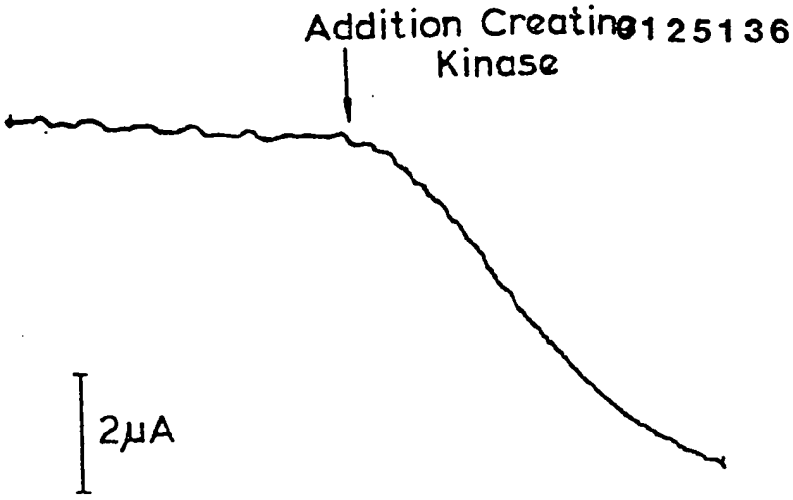
Addition Creatine
Kinase
2μA
6 minutes

FIG. 5.

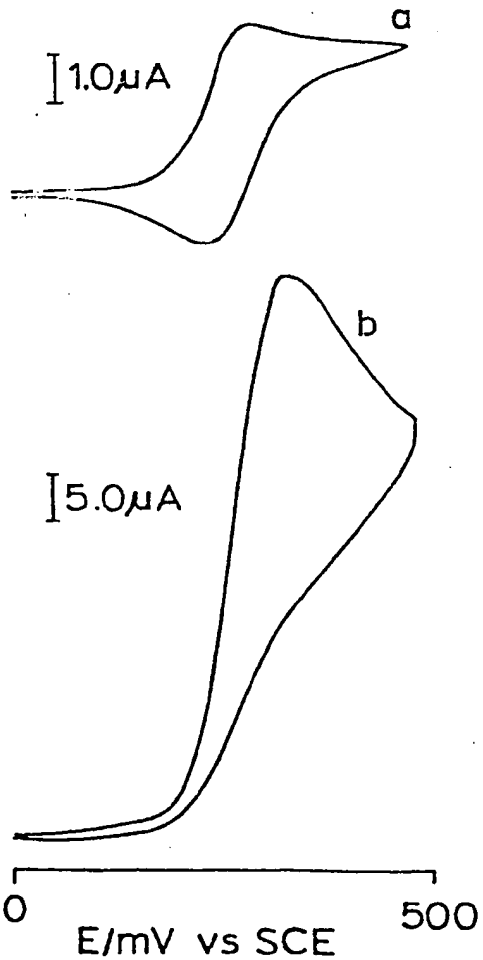
a
1.0μA
b
5.0μA
0
500
E/mV vs SCE

FIG. 6.

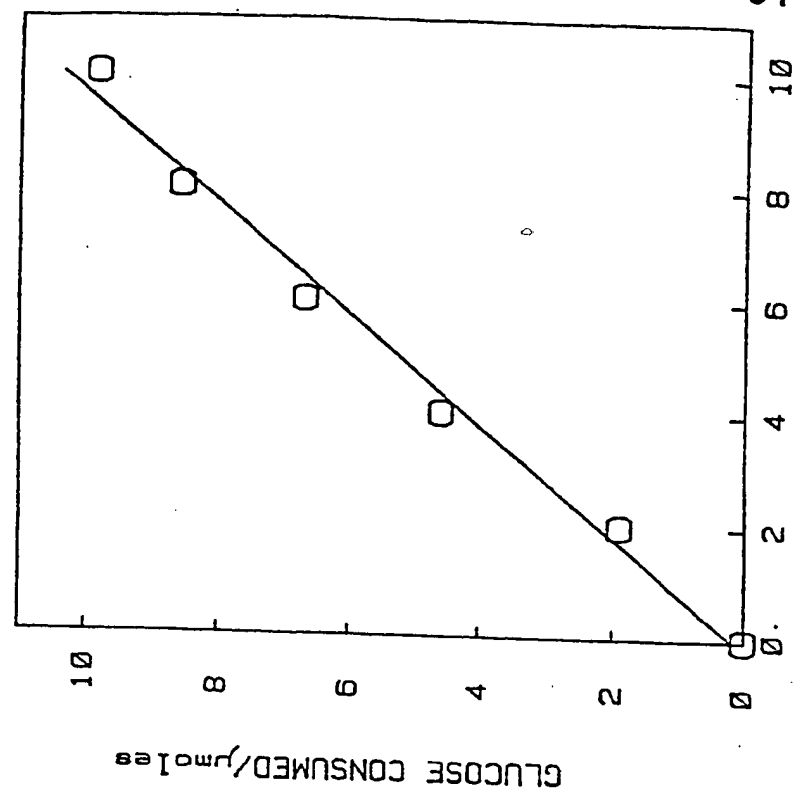
GLUCOSE CONSUMED/μmoles
0
2
4
6
8
10
ATP ADDED/μmoles
0
2
4
6
8
10

FIG. 10.

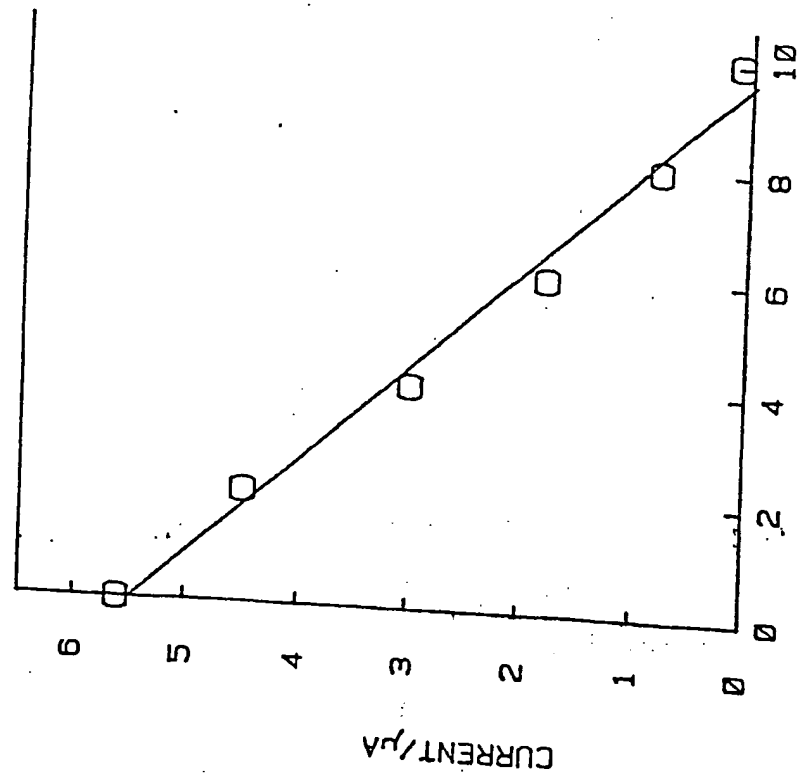
CURRENT/μA
0
1
2
3
4
5
6
ATP/μmoles
0
2
4
6
8
10

FIG. 9.

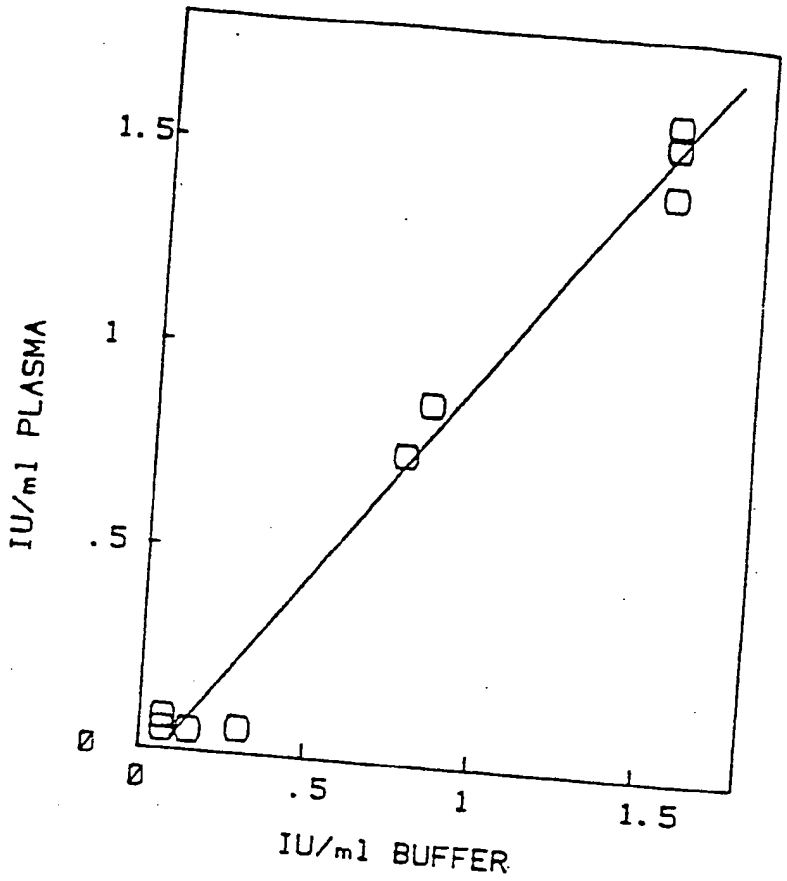

FIG.13.